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Prevention of hepatitis C virus infection in chimpanzees after antibody-mediated *in vitro* neutralization

(neutralizing antibodies/polymerase chain reaction)

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ABSTRACT Hepatitis C virus (HCV) is the most important etiologic agent of non-A, non-B hepatitis and is a major cause of chronic liver disease and hepatocellular carcinoma. Development of an effective vaccine would be the most practical method for prevention of the infection, but whether infection with HCV elicits protective immunity in the host is unclear. Neutralization of HCV *in vitro* was attempted with plasma of a chronically infected patient, and the residual infectivity was evaluated by inoculation of eight seronegative chimpanzees. The source of HCV was plasma obtained from a patient during the acute phase of posttransfusion non-A, non-B hepatitis, which had previously been titered for infectivity in chimpanzees. Neutralization was achieved with plasma obtained from the same patient 2 yr after the onset of primary infection but not with plasma obtained 11 yr later, although both plasmas contained antibodies against nonstructural and structural (including envelope) HCV proteins. Analysis of sequential viral isolates from the same patient revealed significant genetic divergence as early as 2 yr after infection. However, the HCV recovered from the patient 2 yr after the infection had a striking sequence similarity with the HCV recovered from one of the chimpanzees inoculated with the acute-phase virus, suggesting that the progenitor of the new strain was already present 2 yr earlier. This evidence, together with the different sequences of HCV recovered from the chimpanzees that received the same inoculum, confirms that HCV is present *in vivo* as a quasispecies. These results provide experimental evidence *in vivo* that HCV infection elicits a neutralizing antibody response in humans but suggest that such antibodies are isolate-specific. This result raises concerns for the development of a broadly reactive vaccine against HCV.

Hepatitis C virus (HCV) is the most important cause of posttransfusion and community-acquired non-A, non-B hepatitis (1, 2). It is a recently discovered RNA virus that has been classified within the *Flaviviridae* family (3). Infection with HCV is a serious public health problem because it is a major cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (4, 5). Chronic hepatitis C develops in >50% of acutely infected individuals (6). These epidemiologic and clinical features indicate that control of HCV infection demands effective prevention strategies, preferably the development of a broadly reactive vaccine. In recent years, however, concerns have been raised about the degree of protective immunity elicited by HCV infection in the host (7). In addition to the high rate of progression to chronicity (6), chronic hepatitis C was found to be associated with continuous viral replication (8), which suggests that, in most patients, the immune response fails to mediate resolution of the infection. Further concerns emerged from the reanalysis of a series of cross-challenge experiments in chimpanzees (9, 10), which demonstrated that reinfection with either homologous or heterologous strains of HCV can occur. Moreover, such reinfections were sometimes associated with the development of chronic HCV infection. Similar evidence has been obtained recently in β-thalassemic children (11).

Several hypotheses may account for the apparent lack of protective immunity to HCV. These involve the role of host factors, such as the inability to mount a protective immune response against HCV, and viral factors, such as the remarkable degree of genetic heterogeneity of the virus (12–15). HCV, like other RNA viruses, mutates rapidly (16, 17), leading to the simultaneous coexistence of multiple genotypes in the same individual (18) (quasispecies) and, possibly, to the generation of neutralization escape mutants (19).

To address the question of whether protective immunity is elicited by HCV infection, we investigated whether infection with HCV can elicit a neutralizing antibody response in the natural host. For this purpose, we have attempted to neutralize HCV *in vitro* with serum from a chronically infected individual (8, 20) and then to test for residual infectivity by i.v. inoculation of seronegative chimpanzees. As a potential source of neutralizing antibodies, we chose plasma from a patient with chronic HCV infection (8) because previous studies showed that acute self-limited HCV infection, in both chimpanzees (9, 10, 21) and humans (11), does not confer protective immunity, even against reinfection with the homologous virus. Moreover, recent experiments with human immunodeficiency virus (HIV) have documented that protection of a naive chimpanzee could be achieved by *in vitro* neutralization of HIV with serum from a chimpanzee chronically infected with HIV (22).

MATERIALS AND METHODS

Anti-HCV Testing. Antibodies to HCV (anti-HCV) were determined in the human plasmas from patient H with a commercially available second-generation ELISA (Ortho Diagnostic). In addition, antibodies to six target sequences derived from the structural [nucleocapsid, envelope 1 (E1)] and nonstructural (NS) regions (NS3, NS4, and NS5) of the prototype HCV-1 were measured by David Chien (Chiron) using a strip immunoblot assay (RIBA TM; Chiron), as described (23). Antibodies to the putative second envelope (E2) protein, formerly called NS1, were measured against a protein expressed in Chinese hamster ovary (CHO) cells.

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Abbreviations: HCV, hepatitis C virus; E1 and E2, envelope 1 and 2; CID_{50}, 50% chimpanzee infectious doses; ALT, alanine aminotransferase; HIV, human immunodeficiency virus; NS, nonstructural.

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Antibodies against structural and NS proteins of HCV were assayed in chimpanzee sera with second-generation ELISA (manufactured by Ortho Diagnostic or by Abbott) on serum samples obtained weekly for 6 mo after inoculation.

PCR. The PCR was done as a two-step reaction (8) with two sets of nested primers, one derived from the fourth NS (NS4) gene region (24) and the other from the E1/E2 region of the HCV genome (10). Total RNA was extracted from 100 μl of the serum or plasma sample by the guanidinium–phenol–chloroform method, as reported (8).

Direct DNA Sequencing. Selected samples obtained from each chimpanzee (*Pan troglodytes*) 2 and 3 weeks after inoculation were subjected to RNA extraction, and this RNA was reverse-transcribed and amplified with a set of primers that span part of the E1/E2 regions of the HCV genome (10). The PCR products amplified from the E2 region of the HCV genome were purified by GeneClean (Bio 101) and sequenced by the dideoxynucleotide chain-termination method with phage T7 DNA polymerase (Sequenase; United States Biochemical), as described (25). This region contains a hypervariable region that can be used to distinguish individual viral isolates (26, 27). Data were analyzed with GENALIGN (28) for multiple sequence alignment and CLUSTAL (29) for dendrogram construction.

Source of HCV. The virus stock of HCV used for challenge was derived from a plasmapheresis unit obtained during the early-acute phase of posttransfusion hepatitis (on July 12, 1977) from a patient (H) who underwent open-heart surgery (20). This plasma, designated H77, contained $10^{6.5}$ 50% chimpanzee infectious doses (CID_{50}) of HCV per ml, as shown by titration in chimpanzees (ref. 30; R.H.P., unpublished data).

Source of Antibodies for Neutralization. Two plasma samples were used as potential sources of neutralizing antibodies. The first was plasma collected from patient H on August 1, 1990, 13 yr after the acute hepatitis, when he was chronically infected with HCV (8). During this interval, virus replication could be documented repeatedly by the detection of HCV RNA in serum by the PCR (8). This plasma, designated H90, was positive for antibodies to the nucleocapsid (core), E1, E2, NS3, NS4, and NS5 proteins of HCV. The second plasma used as a source of potential neutralizing antibodies was a plasmapheresis unit obtained from patient H on April 16, 1979, 2 yr after the onset of primary infection (20). This plasma, designated H79, had detectable HCV RNA and was positive for antibodies to core, E1, E2, NS3, NS4, and NS5. It was chosen because it was available in sufficient quantity, was positive for antienvelope antibodies at high titers, and was shown to inhibit replication of HCV in studies of viral propagation in cell culture (31). Plasma from a normal blood donor, negative for all antibodies to HCV, whose blood repeatedly failed to transmit hepatitis after transfusion (H.J.A., unpublished data) was used as a negative control.

***In Vitro* Neutralization Test.** The neutralization test used was similar to that used by Emini *et al.* (22) with some modifications. Each plasma was diluted 1:5 in phosphate-buffered saline (PBS), pH 7.4, and then heat-inactivated at 56°C for 30 min before use. One vial of a dilution (in fetal bovine serum) of the challenge virus containing 3200 CID_{50} was further diluted 1:5 in ice-cold PBS, pH 7.4, and then two additional 1:10 dilutions were made in cold PBS/20% fetal bovine serum to yield samples containing 640, 64, and 6.4 CID_{50}, respectively. The *in vitro* neutralization test was done by mixing the virus inoculum (1 ml) with one of the inactivated plasmas (1 ml). The virus/plasma mixtures were incubated overnight at 4°C. Each mixture (2 ml) was then inoculated into one seronegative chimpanzee (Table 1).

Chimpanzees. A total of eight chimpanzees were included in this study. The animals were caged individually and maintained under conditions that met all relevant requirements for the use of primates in an approved facility. None of the eight chimpanzees had signs of active or past HCV infection, as measured by PCR and antibody testing. At the time of the study, all chimpanzees were negative for hepatitis B surface antigen and had normal serum levels of hepatic enzymes. Serum HCV RNA was determined in serial serum samples obtained at intervals of 1, 2, or 4 weeks, during an observation period of 24 weeks after the virus challenge.

RESULTS AND DISCUSSION

Four animals were included in the first neutralization experiment (Table 1). Two of them (87A02 and 1384) were inoculated with 640 and 64 CID_{50}, respectively, mixed with the H90 plasma at a final dilution of 1:10, and two animals (1434 and 1451) were inoculated with 64 and 6.4 CID_{50}, respectively, mixed with the negative control plasma. The latter two chimpanzees served as controls of the neutralization step and as an internal titration of the virus dose. All four chimpanzees developed classical hepatitis C after inoculation (Fig. 1*A*), regardless of the plasma used for neutralization (Table 1). Serum HCV RNA was first detected within 1 week after inoculation in three animals and within 2 weeks in the fourth animal (1451). In the two animals (87A02, 1384) inoculated with 640 and 64 CID_{50} of HCV, respectively, mixed with the H90 plasma, ALT levels were considerably lower than in the two control animals (1434 and 1451) (Fig. 1*A*). Chimpanzee 87A02 did not develop antibody seroconversion, and the infection did not become chronic (Fig. 1*A*). In contrast, antibodies to HCV, as measured by second-generation assay, were detected in the remaining three animals (1384, 1434, and 1451), and in these three animals, the infection became chronic (Fig. 1*A*). Coded liver biopsy specimens from all four animals were analyzed (Fig. 1*A*). Even though the magnitude of ALT elevation varied considerably in experimental and control chimpanzees, the severity of the histologic necroinflammatory changes was virtually the same in all infected animals.

To prove that the HCV recovered from the challenged animals was derived from the virus stock used for the challenge (H77) and not from the virus present in the heat-inactivated plasma (H90) used as a potential source of neutralizing antibodies, sequence analysis of a portion of the E1/E2 region of the HCV genome was done on selected samples recovered from the chimpanzees 2 and 3 weeks after inoculation. The nucleotide sequences of the hypervariable region in the E2 gene of HCV recovered after challenge in the four animals are shown in Fig. 2. In chimpanzees 87A02 and 1384, inoculated with the H77 virus mixed with heat-inactivated H90 plasma, the virus recovered 2 weeks after challenge was derived from H77 and not from H90, on the basis of the analysis of 469 nt including the hypervariable region of E2 (Table 2 and Fig. 2).

The failure of the chronic-phase plasma (H90) to neutralize HCV in the acute-phase plasma (H77) could be explained by the failure of the host to produce neutralizing antibodies.

Table 1. Neutralization experiments

Inoculum number	Chimpanzee number	Virus, CID_{50}	Antiserum (final conc.)
Exp. A			
1	87A02	640	H90 plasma (1:10)
2	1384	64	H90 plasma (1:10)
3	1434	64	Normal plasma (1:10)
4	1451	6.4	Normal plasma (1:10)
Exp. B			
5	1442	64	H79 plasma (1:10)
6	1484		H79 plasma (1:10)
7	1441	64	Normal plasma (1:10)
8	1443	6.4	Normal plasma (1:10)

The table indicates the virus and antisera used in the attempt to neutralize HCV *in vitro*. conc., Concentration.

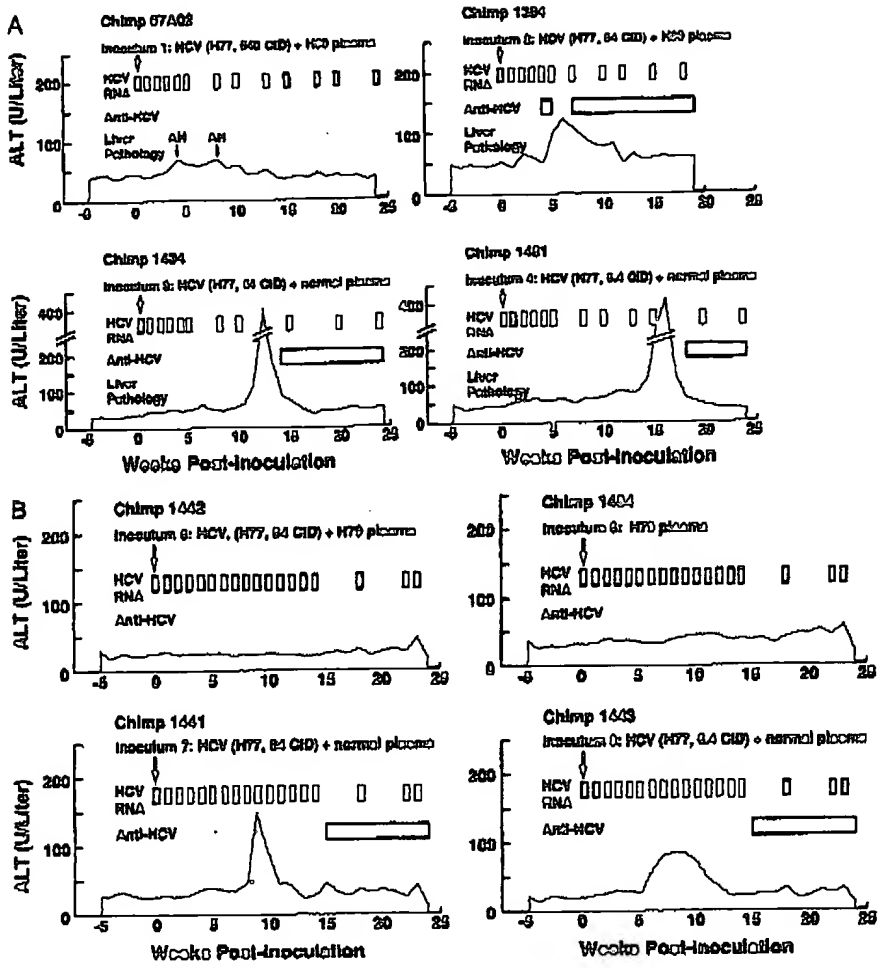

FIG. 1. Course of HCV infection in chimpanzees after antibody-mediated *in vitro* neutralization. Neutralization of HCV was attempted with heat-inactivated plasma from the same patient during the late-chronic phase (strain H90), 13 yr after the initial infection (*A*), or during the early-chronic phase (strain H79), 2 yr after the initial infection (*B*). Upper arrows indicate time of challenge. The gray areas indicate the values of serum ALT. Normal ALT values in chimpanzees range between 6 and 38 units (U)/liter. Open bars (□) indicate negative results for serum HCV RNA by PCR; solid bars (■) indicate positive results. The horizontal bar indicates the time during which serum was positive for antibodies to HCV, as detected by second-generation ELISA assay. Liver pathology: the lower arrows indicate the time and the results—acute hepatitis (AH)—of liver biopsies.

Alternatively, we can postulate that the neutralizing antibodies were produced but lost over time or that the neutralizing ability of such antibodies was isolate-restricted. That the neutralizing antibodies may have changed in response to the genetic variability of the virus is supported by the remarkable sequence variation of the H strain of HCV over 13 yr of chronic infection (16). Comparison of the nucleotide sequences of H77 and H90 strains demonstrated a sequence divergence of 28.2% in a 39-nt domain that included part of the hypervariable region of the E2 protein (16). A high degree of heterogeneity in the hypervariable region was already detected as early as 2 yr after the primary infection (H79 strain) (Fig. 2). This finding prompted us to undertake a second neutralization experiment, in which, as a source of

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          1150                                                                1231
H77-1     GAAACCCACGTCACCGGGGGAAATGCCGGCCGCACCACGGCTGGGCTTGTTGGTCTCCTTACACCAGGCGCCAAGCAGAAC
H77-2     .....G.....
H79        .....G.....A....G.....A...C.A..T..T..G.....C.....
H90        .....G.....T..GT.CT....A...C.A..T.....G....C.....
C87A02     .....G.....TA.....CT..AC.....
C1384      .....G.....TA.....CT..AG.....
C1434      .....G.....TA.....CT...C.....
C1451      .....G.....T.A....G.....A...C.....T.....T.....
C1441      .....G.....A.....
C1443      .....
C1304      .....A.....
HCV-1      .....G.....A...TGT.T....AT.....A.C.....CG.....
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FIG. 2. Multiple nucleotide sequence alignment of 81 nt representing the hypervariable region of the E2 gene, spanning map positions 1150–1231 of the HCV genome (32). H77-1 and H77-2 (16) denote two independent sequences obtained in our laboratory from the same sample of H77, collected from patient H on July 12, 1977; H79 denotes the sample obtained from patient H on April 16, 1979, during the early-chronic phase; H90 denotes the sample obtained from patient H, on August 1, 1990, during the late-chronic phase (16); C87A02, C1384, C1434, and C1441 denote the samples obtained 2 weeks after inoculation from the respective chimpanzees; C1451 and C1443 denote the samples obtained 3 weeks after inoculation from the respective chimpanzees; C1304 denotes the sample obtained 9 weeks after inoculation from the respective chimpanzee infected with undiluted H77 ($10^{4.5}$ CID_{50}) (33) and included for sequence comparison; HCV-1 denotes the prototype HCV strain (32), included as a reference. The remaining sequence of the 469 nt spanning map position 854–1323, which is not shown, is available upon request. When microheterogeneity was detected, the conserved nucleotide was used for sequence comparison. Dots indicate nucleotide identity with the H77-1 sequence.

Table 2. Percentage nucleotide sequence identity of 12 HCV isolates

Strain	H77-1	H77-2	H79	H90	C87A02	C1384	C1434	C1451	C1441	C1443	C1304	HCV-1
H77-1		99.6	95.5	93.4	97.7	96.8	97.7	96.2	98.5	99.6	98.9	92.3
H77-2		—	95.9	93.8	97.7	96.8	97.7	96.6	98.9	99.4	98.7	92.8
H79			—	95.7	95.5	94.5	95.7	97.7	95.7	95.3	95.5	92.3
H90				—	93.6	92.5	93.8	94.0	93.0	93.2	92.8	90.8
C87A02					—	98.9	99.6	96.6	97.0	97.2	97.0	92.5
C1384						—	98.5	95.5	96.6	96.4	96.2	92.1
C1434								96.8	97.0	97.2	97.0	92.3
C1451								—	96.4	95.9	96.2	91.3
C1441										98.3	98.5	92.3
C1443										—	98.9	92.1
C1304											—	92.1
HCV-1												

The percentage identity was based on 469 nt spanning map positions 854–1323 (32) derived from a portion of the E1 and E2 genes, including the hypervariable region. The symbols for the HCV isolates are the same as those described for Fig. 2.

potential neutralizing antibodies, we used plasma obtained from patient H 2 yr after the onset of primary infection (H79) (20). The neutralization protocol was essentially the same as that used in the first experiment.

Four animals were included in the second neutralization experiment (Table 1). One chimpanzee (1442) was inoculated with 64 CID_{50}, mixed with the H79 plasma (at a final dilution of 1:10), two (1441 and 1443) were inoculated with 64 and 6.4 CID_{50}, respectively, mixed with the negative control plasma, and one was inoculated with heat-treated H79 plasma alone to monitor the adequacy of the inactivation step. The clinical course of HCV infection in the four chimpanzees is shown in Fig. 1*B*. We did not detect any virologic, biochemical, or serologic evidence of HCV infection in the animal (1442) inoculated with the H77 virus mixed with the inactivated H79 plasma, indicating that the *in vitro* neutralization had abrogated the HCV infectivity. Similarly, chimpanzee 1484, which received the inactivated H79 plasma alone, did not develop any virologic or biochemical signs of HCV infection. Neither of the two animals developed antibodies against structural or NS proteins of HCV. In contrast, hepatitis C developed in the two animals (1441 and 1443) inoculated with the H77 virus mixed with the negative control plasma (Fig. 1*B*). Viremia first appeared within 1 or 2 weeks after inoculation and remained persistently detectable in one animal (1441) throughout the observation period. The two animals also developed biochemical evidence of hepatitis, and both seroconverted. The serum ALT levels in the two control animals (1441 and 1443) were considerably lower than those observed in the two control animals (1434 and 1451) of the previous experiment (Fig. 1*A*), which received the same CID_{50} of virus challenge (Table 1).

A comparative sequence analysis between the virus used for the inocula and the viruses recovered from the chimpanzees was undertaken. The analysis, based on the sequence of 469 nt comprising a portion of the E1 and E2 genes (including the hypervariable region), demonstrated that none of the sequences recovered from the chimpanzees after the viral challenge was identical to the consensus sequence of the HCV strain used for inoculation; the sequences differed from each other at 2–21 sites. In the two chimpanzees (87A02 and 1384) that received the H77 virus mixed with the inactivated H90 plasma, the viruses recovered 2 weeks after challenge exhibited 11 and 15 nucleotide substitutions, respectively, when compared with the sequence of H77 virus used for challenge. Comparison of additional sequences from chimpanzees 87A02 and 1384 3 weeks after inoculation (data not shown) showed 100% identity with the nucleotide sequences detected 1 week earlier (2 weeks after inoculation). These data suggest that the degree of heterogeneity within the hypervariable region of HCVs recovered from chimpanzees that received the same inoculum reflects the presence of a quasispecies in the original H77 inoculum, rather than an exceedingly rapid mutation rate of the predominant HCV strain. In control animals, sequence comparison with the original infecting virus (H77) showed a degree of heterogeneity that ranged from 2 to 18 nucleotide substitutions [chimpanzee 1434 had 11 nucleotide substitutions, chimpanzee 1451 had 18 substitutions, chimpanzee 1441 had 2 substitutions, and chimpanzee 1443 had 7 substitutions, although none of these 7 nucleotide substitutions was in the hypervariable region (Fig. 2)]. It is interesting to note that, although all animals received the same challenge virus (H77) at different doses, the viral sequences recovered from the four control chimpanzees 1 week after the first appearance of viremia all differed (Table 2). Moreover, the virus recovered from chimpanzees given the same dose of challenge virus differed considerably, indicating that the degree of heterogeneity was not solely affected by viral dose. The nucleotide sequences of an 81-nt region containing the hypervariable region of the different HCV isolates are shown in Fig. 2. A dendrogram of the genetic relatedness of the sequenced 469-nt region of the HCV isolates studied is shown in Fig. 3. The nucleotide sequence similarities among the viruses recovered from the chimpanzees inoculated with HCV (strain H77) ranged from 95.5 to 99.6%, whereas the nucleotide similarities with the HCV inoculum ranged from 96.2 to 99.6% (Table 2). These data strongly suggest the existence of a quasispecies in the original inoculum, confirming previous observations in infected patients (18).

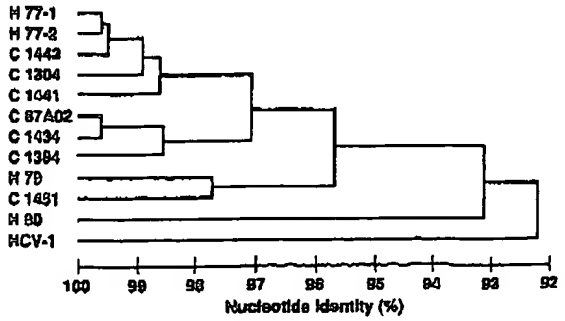

FIG. 3. Dendrogram of genetic relatedness of 12 HCV isolates, based on nucleotide identity of 469 nt spanning map positions 854–1323 (32) derived from a portion of the E1 and E2 genes, including the hypervariable region. This dendrogram was constructed by the program CLUSTAL (29), with a limit of 25 sequences and a gap penalty of 0. The scale showing percentage identity was based upon manual calculation. Symbols for the HCV isolates are the same as those of Fig. 2.

Our study provides experimental evidence *in vivo* that infection with HCV elicits a neutralizing antibody response that can prevent HCV infection in chimpanzees. However, neutralizing antibodies against the acute-phase virus (H77) were detected only during the early chronic phase, 2 yr after the initial infection, but not 11 yr later, suggesting that HCV elicits a restricted neutralizing antibody response. By analysis of the HCV sequences recovered from chimpanzees inoculated with the acute-phase virus, we demonstrated that HCV circulates *in vivo* as a quasispecies, as suggested (13). In one chimpanzee inoculated with the acute-phase virus, we recovered a relatively divergent HCV genotype. Interestingly, this virus had a striking sequence similarity to that recovered from patient H 2 yr after infection, suggesting that the progenitor of the variant that emerged as the predominant strain 2 yr after the initial infection was already present during the acute phase. These data indicate that HCV, like other RNA viruses (34), is not present in a patient as a single virus species but as a population of multiple related variants. The coexistence of a mixed viral population may lead to the rapid emergence of viruses that escape neutralization by the immune system; this could be a strategy whereby HCV evades the host's immune surveillance and establishes persistent infection in a high proportion of infected individuals. Although the principal neutralization determinant(s) of HCV still remains to be defined, multiple lines of evidence (19, 35) suggest that it may correspond to the hypervariable region contained in the E2 protein. The potential importance of this hypervariable region is also underscored by the strong analogy it shares with the V3 loop of HIV, which represents the principal neutralizing domain (36). Recently, Choo *et al.* (37), using recombinant envelope proteins of HCV, reported successful vaccination of chimpanzees against the homologous strain of HCV, but whether recombinant envelope proteins could protect against challenge with a heterologous virus remains to be shown. A successful vaccine must protect against multiple viral isolates. The extent to which cellular immunity operates in the mechanism of protective immunity against HCV, as recently proposed for HIV (38), is still undefined. Regardless of the mechanism, the high degree of genetic heterogeneity of HCV *in vivo*, as manifested both by the generation of a viral quasispecies and by the continuous emergence of neutralization escape mutants, may represent an obstacle to the development of a broadly reactive vaccine for the control of HCV infection.

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